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(54) HCV-DERIVED RNA POLYMERASE GENE

(57) The present invention provides a gene encoding an RNA polymerase which plays an important role in the reproduction of hepatitis C virus, and a method of screening using this gene or this RNA polymerase protein, thereby allowing easy performance of screening for substances which inhibit the RNA polymerase playing an important role in HCV reproduction.

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Description

Technical Field

[0001] The present invention relates to an RNA polymerase gene derived from hepatitis C virus (referred to as "HCV" herein), a method of screening using this gene or this RNA polymerase protein, and a substance able to be isolated by this screening method.

Prior Art

[0002] Generally known viral hepatitis includes hepatitis A which is mainly orally transmitted, and hepatitis B transmitted by means of the blood. Moreover, apart from these hepatitis, there is hepatitis called non-A, non-B hepatitis which is transmitted by means of blood transfusion. Since most of these infected with non-A, non-B hepatitis become chronic, and the incidence of development into cirrhosis and hepatoma is high, this is one disease for which the establishment of a certain means of treatment is urgently sought.

[0003] Through the causative agent of non-A, non-B hepatitis had been unclear for a long time, recently the causative virus was isolated by M. Houghton et al. (Japanese Patent Application Laid-Open (Kohyo) No. 2-500880), and was termed "HCV". HCV is a single-stranded RNA virus belonging to the Flavivirdae, the length of its whole genomic RNA is about 9.4kb. The genomic RNA is divided into 7 regions; core, E1, E2/NS1, NS2, NS3, NS4, and NS5; and the genes related to virus growth, etc. are primarily included in downstream regions from NS3.

[0004] HCV RNA polymerase is related to the transcription and replication of genomic RNA, and plays an important role in the reproduction of HCV. The gene encoding this polymerase is thought to be included in the above-mentioned NS5 region (Z. H. Yuan et al., Biochemical and Biophysical Research Communications 232, 231-235(1997), S. B. Hwang et al., Virology 227, 439-446 (1997), S. E. Behrens et al., The EMBO Journal 15 12-22(1996)).

The problem to be solved by the invention

[0005] If the gene encoding HCV RNA polymerase can be isolated, it will become possible using this gene to easily screen for substances inhibiting RNA polymerase, and contribute greatly to the development of drugs for treating HCV. However, at present, although the nucleotide sequence of a portion of the NS5 region has been clarified (Japanese Patent Application Laid-Open (Kokai) No. 6-225770), the entire nucleotide sequence of the RNA polymerase gene has yet to be clarified.

[0006] The object of the present invention is to isolate the gene encoding the full length of HCV-derived RNA polymerase, to determine its nucleotide sequence, as well as to establish its expression system.

[0007] A further object of the present invention is to provide a screening method for a substance which inhibits the activity of this gene or this protein employing this gene or this RNA polymerase protein.

Means for solving the problem

[0008] In order to solve the above problem, the present inventors, as result of deliberate and focused research have succeeded in isolating the gene encoding the full-length of HCV-derived RNA polymerase, thereby completing the present invention.

[0009] That is to say, the present invention relates to the following (1) to (3) .

(1) A gene encoding the following protein (a) or (b) :

(a) a protein consisting of the amino acid sequence represented in SEQ ID NO: 2;
(b) a protein consisting of an amino acid sequence derived from the amino acid sequence represented in SEQ ID NO: 2 by deletion, substitution or addition of one or several amino acid(s), and which has RNA polymerase activity.

(2) A method of screening a substance which inhibits the activity of the gene of (1) above, or of the protein consisting of the amino acid sequence represented in SEQ ID NO: 2, wherein this method comprises the following steps:

(a) a step of contacting the gene of (1) above or the protein consisting of the amino acid sequence represented in SEQ ID NO: 2, or a fragment of this protein, with a test sample; and,
(b) a step of selecting a substance which inhibits the activity of the gene of (1) above, or of the protein or the partial peptide fraction consisting of the amino acid sequence represented in SEQ ID NO: 2.

(3) A substance able to be isolated by the method of (2) above, wherein this substance inhibits the activity of the gene of (1) above or of the protein consisting of the amino acid sequence represented in SEQ ID NO: 2.

[0010] The descriptions contained in the specification of Japanese Patent Application No. 10-177817, which forms the basis of the right of priority of the present application, are incorporated herein in their entirety.

Disclosure of the Invention

[0011] Below, the present invention will be

explained in detail.

[0012] The gene of the present invention encodes (a) a protein consisting of the amino acid sequence represented in SEQ ID NO: 2; or, (b) a protein consisting of an amino acid sequence derived from the amino acid sequence represented in SEQ ID NO:2 by deletion, substitution, or addition of one or several amino acid(s) and having RNA polymerase activity.

[0013] The deletion, etc. of one or several amino acid can be performed by techniques in common use at the time of filing this application, such as, for example, site-specific mutagenesis (Nucleic Acids Res. 10, 6487-6500, 1982) .

[0014] The gene of the present invention is able to be obtained from the blood of non-A, non-B hepatitis patients as described in the examples, or from the strain of *E. coli* into which a vector (pCALN/HCV RBZ) comprising the gene of the present invention was introduced, has been deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (1-1-3, Higashi, Tsukuba-shi, Ibaraki-ken, Japan) (Accession No. FERM BP-6763) on October 31, 1997) .

[0015] Further, the present invention relates to a screening method for a substance which inhibits the activity of this gene or this protein, employing the gene of the present invention or an RNA polymerase protein consisting of the amino acid sequence represented in SEQ ID NO: 2; and, to a substance able to be isolated by this screening method employing this gene or this RNA polymerase protein.

[0016] The RNA polymerase encoded by the gene of the present invention is an enzyme involved in the transcription and replication of HCV genomic RNA. Therefore, a substance which inhibits this enzyme is thought to be able to prevent the reproduction of HCV, and is promising as a drug for treating non-A, non-B hepatitis. By using the gene of the present invention it will be possible to produce HCV-derived RNA polymerase easily and in great quantities, and as a result of this, the screening of inhibitory substances for the RNA polymerase will become simpler.

[0017] The protein of the present invention that can be used for screening can be either a recombinant type, a wild type, or a partial peptide. Further it can be a purified peptide or a partial peptide thereof.

[0018] One embodiment of this method of screening comprises the steps of (a) contacting the gene of the present invention or the protein consisting of the amino acid sequence represented by SEQ ID NO: 2, or a fragment of this protein, with a test sample; and, (b) selected a substance which inhibits the activity of the gene of the present invention or the protein consisting of the amino acid sequence represented by SEQ ID NO: 2. There is no particular limitation on what can be used as a test material in this screening method but for example, a cell extract, a cell culture supernatant, a protein, a peptide, or synthetic low molecular weight compound can be used.

Examples

(1) Isolation of HCV RNA from the blood of a hepatitis patient and PCR amplification of the isolated RNA

[0019] RNA was extracted from the blood of a non-A, non-B hepatitis patient by guanidine thiocyanate and phenol/chloroform method and RT-PCR was performed according to the method described in Japanese Patent Application Laid-Open (Kokai) No. 6-225770. Using this cDNA as a template, PCR (Science 230:1350 (1985)) was performed using the primers described in Japanese Patent Application Laid-Open (Kokai) No. 6-225770, and four types of amplification fragment (C6-62 region, C6-66 region, C6-79 region, C6-82 region) were obtained. These amplification fragments were cloned using cloning vector pBM, and the nucleotide sequences of the amplification fragments were determined by Sanger's dideoxy- termination procedure (Science, 214, 1205(1981)). It should be noted that cloning vector pBM is a vector that was constructed such that mutations do not occur easily, taking into consideration the nature of the HCV gene to incorporate mutations easily during replication and cloning. (Japanese Patent Application Laid-Open (Kokai) No. 6-225770) .

[0020] The position of each amplification fragment was determined by a comparison of the homology of the amplification fragments within the clones obtained by the above method, and the previously reported non-A, non-B hepatitis virus gene, each of these amplification fragments were joined together by PCR, and the desired DNA fragment (amplified DNA) was cloned into a vector. pCALN/HCV RBZ was prepared using this cloning vector.

(2) Determining the nucleotide sequence of the fragment encoding NS5B

[0021] The fragment encoding NS5B was amplified by PCR with pCALN/HCV RBZ as a template using the following primers.

PCR primers:

[0022]

NS5B1 5' -ATC CCT CGA GAT GTC CTA CAC ATG GAC AGG-3 (SEQ ID NO:3)
NS5B2 5' -TAT GGA TCC AAG CTT CAC CGG TTG GGG AGC AGG T-3' (SEQ ID NO:4)

[0023] The reaction solution was prepared by adding to a 0. 5ml tube, 10 µl 10X PCR buffer II (500mM KCl, 100mM Tris-HCl pH8.3, 15mM $MgCl_2$), 16 µl of 1.25mM dNTP, 5 µl each of the 2 types of primers (20 µM) (NS5B1, NS5B2), 0.5 µl of 1 units/µl AmpliTaq DNA

Polymerase (PERKIN ELMER) and adjusting to 100 µl with sterilized water. Thermal conditions were set such that, after initial heating at 95°C for 5 minutes, 25 cycles were conducted with conditions of 95°C for 1 minute for denaturation, 55°C for 1 minute for annealing, and 72°C for 3 minutes for extension, and thereafter was finally maintained at 72°C for 10 minutes. A portion of the post-reaction solution was subjected to agarose gel electrophoresis, and the specifically amplified DNA fragment was confirmed.

[0024] After purifying this DNA fragment according to the Gene Clean (Biolol) method, the fragment was digested with XhoI and BamHI. The digested reaction solution was subjected to agarose gel electrophoresis, the desired DNA fragment was extracted from the gel, and the concentration of DNA in the extract was measured. The cloning vector pET-15b (Novagen) was similarly digested with XhoI and BamH, then purified. The digested reaction solution was subjected to agarose gel electrophoresis, the desired DNA fragment was extracted from the gel and the concentration of DNA in the extract was measured.

[0025] In respect the above-mentioned two types of DNA fragment extracted from the gel, a ligation reaction was performed according to the DNA ligation Kit ver.2 (Takara) method, and *E. coli* was transformed using a portion of the reaction solution. The transformed strain thereby obtained was cultured overnight on an LB-Amp plate(1% bactotryptone, 0.5% yeast extract, 1% NaCl, 1.4% agar, ampicillin 100 µg/ml). Thereafter, the colonies appearing on the plate were each cultured (37°C, 16 hours) with a tube containing 2ml LB-Amp medium (1% bactotryptone, 0.5%yeast extract , 1% NaCl, ampicillin 100 µg/ml) . The cultured fluid was centrifuged to collect the microorganism and plasmid DNA was extracted by mini-preparation method. The plasmid was digested with XhoI and BamHI, and then the digested product was subjected to agarose gel electrophoresis, and a plasmid clone into which the desired DNA fragment had been introduced, was obtained. This plasmid clone was designated pET-15b HCV pol.

[0026] The plasmid clone pET-15b HCV pol obtained above was digested with XbaI and BamHI, this product solution of the enzyme reaction was subjected to agarose gel electrophoresis and the desired DNA fragment was extracted. Similarly, baculovirus transfer vector pVL1392 (Pharmigen) was digested with XbaI and BamHI, this product solution of the enzyme reaction was subjected to agarose gel electrophoresis, and the desired DNA fragment was extracted.

[0027] By performing a ligation reaction with respect to the above-mentioned two types of DNA fragment extracted from agarose-gel, NS5B gene tagged with 6x His at the N-terminus was introduced into pVL1392. *E. coli* was transformed using this ligation reaction solution. The obtained transformed strain was cultured overnight on LB-Amp plate (1% bactotryptone, 0.5% yeast extract 1% NaCl, 1.4% agar, ampicillin 100 µg/ml), thereafter each colony appearing on the plate was cultured (37°C , 16 hours)with a tube containing 2ml LB-Amp medium (1%bactotryptone, 0.5% yeast extract , 1% NaCl, ampicillin 100 µg/ml). After the cultured fluid was centrifuged, the plasmid DNA was mini-prepped and DNA solution was prepared. After digested with XbaI and BamHI, the product solution of the enzyme reaction was subjected to agarose gel electrophoresis, and a clone into which the desired DNA fragment had been introduced was obtained. This clone was designated pVL1392 His HCV pol. Using pVL1392 His HCV pol, a recombinant baculovirus (BacHisH-CVpol) was produced with BaculoGold (Pharmigen). Production of the baculovirus was performed in accordance with the descriptions in "Baculovirus Expression Vector System: Procedures and Methods Manual" (Pharmigen).

(3) Expression and purification of RNA polymerase

[0028] sf21 AE cells were infected with BacHisH-CVpol, with moi=1 or 2, and three days after infection (72 hours later), cells were collected, and extracted with a buffer comprising 10mM Tris/HCl pH7.9, 0.5M NaCl, 1.5mM $MgCl_2$, 7mM 2-ME, 0.1% Triton X-100, 25% glycerol, 1mM PMSF, and 10 µg/ml leupeptin. The whole cell extract was first passed through a Ni-NTA column (QIAGEN, 60mM imidazole), then purified with FPLC (MonoQcolumn , Pharmacia). RNA polymerase was eluted from MonoQ column with a buffer containing 0.1-1.0M NaCl gradient.

(4) Confirmation of RNA polymerase activity

[0029] Preceding measurement of HCV RNA polymerase activity, in order to determine the optimal concentrations for magnesium acetate and KCl reactions, an RNA specific to HCV was produced as an RNA polymerase reaction template.

[0030] HCV cDNA downstream of the PvuII site (9240) of HCV was cloned into Bluescript KSII (+), this vector was designated HCV RNA-9610. The prepared cloning vector Bluescript KSII (+) HCV RNA-9610 was digested with DraI, PstI, NheI, etc. and after purification, was supplied to an *in vitro* transcription reaction using T7 RNA polymerase. Due to the differences in the restriction enzymes that were used; DraI, PstI and NheI; the synthesized RNA differed in length (377nts in the case of DraI, 340nts in the case of PstI, and 305nts in the case of NheI). These RNA were designated HCV RNA-9610, HCV RNA-9576, HCV RNA-9541, respectively. It should be noted that HCV RNA-9610 has two extra UMPs at its 3' terminus.

[0031] With the above-mentioned HCV RNA as a template, the optimal concentration of magnesium acetate in the HCV RNA polymerase activity measurement system was determined. HCV RNA polymerase(MonoQ column fraction) 5/µl was added to 50 µl of buffer

(20mM HEPES/KOH pH7.6, 50mM KCl, 1mM DTT, 25 μg/ml actinomycin D, 0.5mM ATP,CTP,GTP, 50 μM UTP, 5 μCi [α -^{32}p] UTP (15TBq/mmol, Amersham), 10pmole HCV RNA-9541, 400U/ml RNase inhibitor (TOYOBO)), and in this solution, the concentration of magnesium acetate was set at eleven levels within a range of 0-10mM and incubated at 29°C for 1.5 hours. Products were synthesized at each magnesium acetate concentration and subjected to electrophoresis with 4% PAGE/6M urea. The PSL of the product was measured with BAS. As a result, optimal magnesium acetate concentration was 3-4mM.

[0032] Similarly, the optimal concentration of KCl in the HCV RNA polymerase activity measurement system was determined. Here polyA was used as a template instead of HCV RNA. 10 μl of HCV RNA polymerase fraction was added to 50 μl of buffer (20mM HEPES/KOH pH7.6, 5mM magnesium acetate, 1mM DTT, 25 μg/ml actinomycin D, 10 μM UTP, 2.5 μCi [α -^{32}P] UTP(15TBq/mmol, Amersham), 10 μg/ml poly A(Pharmacia), 100 μM UpU(Sigma), 400U/ml RNase inhibitor (Takara)), and the KCl concentration was set at 10 levels within a range of 20-200mM, and the amount of UMP uptake at each concentration was measured. This measurement itself was performed by firstly, by precipitation of the [α-^{32}P] UMP that was taken up within 10% TCA, collection on a glass filter (GF/C, Whatman), and measuring with a liquid scintillation counter (Aloka). With polyA as a template, the optimal salt concentration of KCl in the polymerase activity observation system, in the presence of UpU primer, was 100mM.

[0033] Further, the KCl optimal concentration of the HCV RNA polymerase activity measurement system in the case where HCV RNA-9541 is used as a template, was determined. In the reaction system, the amount of HCV RNA polymerase was set at 5 μl, the magnesium acetate concentration was set at 3.5mM, 10 pmole of HCV RNA-9541 was used as a template but otherwise the reaction system was identical to the experimental system for determining the optimal concentration of magnesium acetate. The concentration of KCl was set at nine levels within a range of 50-200mM. The result of this measurement was that the optimal concentration of KCl was 50mM. Here, the cause of the difference in KCl optimal concentration was presumed to be due to the secondary structure of the template. (5) Inhibition of the uptake of UMP of HCV RNA polymerase by Rabbit anti-HCV RNA polymerase antibodies.

[0034] NS5B (HCV RNA polymerase) within pET-15b HCV pol was expressed in *E. coli*, and then purified with a Ni-NTA (QIAGEN) column. A rabbit was immunized using this protein as an antigen, and antibodies were (anti-HCVpol) produced.

[0035] First, 20 μl of HCV RNA polymerase purified with Ni-NTA and incubated at room temperature for 30 minutes under the conditions of a final concentration of 3.1, 6.3, 12.5, 25, 50 μg/ml of rabbit IgG anti-HCV RNA polymerase; or as a control, normal rabbit IgG. The composition of the buffer that was used was 20mM HEPES/KOH pH7.6, 100mM KCl.

[0036] After incubation, this HCV RNA polymerase solution was re-adjusted such that it was composed of 3mM magnesium acetate, 1mM DTT, 25 μg/ml actinomycin D, 10 μM UTP (Pharmacia), 2.5 μCi [α-^{32}P] UTP (15TBq/mmol, Amersham), 10 μg/ml poly A (Pharmacia), 100 μM UpU (Sigma), 400U/ml Prime RNase inhibitor (5'-3' Inc.), and incubated at 29°C for 1.5 hours. The [α-^{32}P] UMP that was taken up was precipitated in 10% TCA, collected on a glass filter(GF/C, Whatman), and measured with a liquid scintillation counter (Aloka). As a result, inhibition of the uptake of UMP of HCV RNA polymerase by the rabbit anti-HCV RNA polymerase anti-body was observed.

[0037] All publications, patents and patent publications cited herein are incorporated into this specification in their entirety.

Effect of the invention

[0038] The present invention provides a novel RNA polymerase gene derived from hepatitis C virus and a method of screening using this gene or this RNA polymerase protein. Screening of an inhibitory substance of this RNA polymerase can be easily performed by using this gene.

Claims

1. A gene encoding the following protein (a) or (b) :

   (a) a protein consisting of the amino acid sequence represented in SEQ ID NO: 2;
   (b) a protein consisting of an amino acid sequence derived from the amino acid sequence represented in SEQ ID NO: 2 by deletion, substitution or addition of one or several amino acid(s), and which has RNA polymerase activity.

2. A method of screening a substance which inhibits the activity of the gene according to claim 1 or of the protein consisting of the amino acid sequence represented in SEQ ID NO: 2, wherein said method comprises the following steps:

   (a) a step of contacting the gene according to claim 1 or the protein consisting of the amino acid sequence represented in SEQ ID NO: 2, or a fragment of said protein, with a test sample; and,
   (b) a step of selecting a substance which inhibits the activity of the gene according to claim 1 or of the protein consisting of the amino acid sequence represented in SEQ ID NO: 2.

3. A substance able to be isolated by the method according to claim 2, wherein said substance inhibits the activity of the gene according to claim 1 or of the protein consisting of the amino acid sequence represented in SEQ ID NO: 2.

INTERNATIONAL SEARCH REPORT

International application No.
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A. CLASSIFICATION OF SUBJECT MATTER
Int.Cl[6] C12N15/54, C12N9/12, C12Q1/68, C12Q1/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int.Cl[6] C12N15/54, C12N9/12, C12Q1/68, C12Q1/48

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
REGISTRY (STN), CA (STN), MEDLINE (STN), WPI (DIALOG), BIOSIS (DIALOG), GenBank/EMBL/DDBJ, SwissProt/PIR/GeneSeq

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO, 96/37619, A1 (Ist. Ricerche Biol. Molecolare Angeletti), 28 November, 1996 (28. 11. 96) & AU, 9659098, A & EP, 842276, A1 & JP, 10-507370, W & IT, 1278077, B & BR, 9609178, A	1-3
X	LOHMANN, V. et al., "Biochemical properties of hepatitis C virus NS5B RNA-dependent RNA polymerase and identification of amino acid sequence motifs essential for enzymatic activity", J. Virol. (1997) Vol. 71, No. 11, p.8416-8428	1-3
X	WO, 97/12033, A1 (Univ. Emory), 3 April, 1997 (03. 04. 97) & AU, 9672007, A & EP, 859833, A1	1-3
X	AL, R.H. et al., "Expression of recombinant hepatitis C virus non-structural protein 5B in Escherichia coli", Virus Res. (1998. Feb.) Vol. 53, No. 2, p.41-49	1-3

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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Date of the actual completion of the international search	Date of mailing of the international search report
7 September, 1999 (07. 09. 99)	14 September, 1999 (14. 09. 99)
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer
Facsimile No.	Telephone No.

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INTERNATIONAL SEARCH REPORT

International application No.
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	YUAN, Z.H. et al., "Expression, purification, and partial characterization of HCV RNA polymerase", Biochem. Biophys. Res. Commun. (1997) Vol. 232, No. 1, p.231-235	1-3
Y	JP, 6-225770, A (The Tokyo Metropolitan Institute of Medical Science), 16 August, 1994 (16. 08. 94) (Family: none)	1-3

Form PCT/ISA/210 (continuation of second sheet) (July 1992)